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Ability of Yeast Ty-VLPs (Virus-Like Particles) Containing Varicella-Zoster Virus (VZV)gE and Assembly Protein Fragments to Induce In Vitro Proliferation of Human Lymphocytes From VZV Immune Patients

Michael D. Welsh,[1]* David R. Harper,[1] Mercedes Garcia-Valcarcel,[2] Wendy J. Fowler,[2] Celia Aitken,[1] Don J. Jeffries,[1] and Guy T. Layton[2]

[1]*Department of Virology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, London, United Kingdom*
[2]*British Biotech, Ltd., Cowley, Oxford, United Kingdom*

Yeast Ty virus-like particles (VLPs) containing viral protein inserts have previously been shown to be potent immunogens, inducing both humoral and cell mediated immunity (CMI). The antigenicity of hybrid VLPs containing fragments of the varicella-zoster virus (VZV) gE protein or the assembly protein (AP) was assessed by lymphocyte proliferation. Peripheral blood mononuclear cells (PBMCs) from patients with a recent natural VZV infection were stimulated in vitro with VZV-VLPs together with control antigens. PBMC samples from both varicella (85%) and zoster (75%) patients proliferated in responses to at least one of the gE VZV-VLPs. As reported for the first time, VZV specific lymphocyte responses were also identified towards the VZV AP in two varicella and two zoster patient samples. The results demonstrate specific CMI recognition of the VZV gE fragments tested and the VZV AP delivered in the form of recombinant Ty-VLPs, and highlights their potential use as a recombinant antigen delivery system for vaccination. ***J. Med. Virol. 59:78–83, 1999.***

KEY WORDS: *Herpesviridae;* VZV-VLPs; CMI; recombinant; vaccine

INTRODUCTION

Varicella zoster virus (VZV) is a member of the family *Herpesviridae* and is the causative agent of both chickenpox (varicella) and shingles (zoster). Serological evidence reflects the highly infectious nature of VZV [Kangro et al., 1994], which following primary varicella infection becomes latent in spinal ganglia from where it may reactivate to cause shingles. Predisposition to severe VZV infections is due to impairment of cell mediated immunity (CMI) rather than humoral immunity, and factors such as increasing age, disease, or immunosuppressive therapy are associated with reactivation of the latent virus [Arvin, 1992].

A live attenuated VZV vaccine has been available for some time [Plotkin, 1994; Cimons, 1995], and although clinical trials have shown the vaccine to be generally well tolerated [Clements et al., 1995; Kuter et al., 1995], there is evidence of vaccine reactivation from latency causing zoster, and there are doubts with respect to long-term protection provided [Kangro, 1990; Clements et al., 1995]. The development of an effective subunit VZV vaccine would be advantageous, especially for administration to immunocompromised patients [Kangro, 1990], and possibly as a booster vaccine to enhance VZV immunity against shingles [Hayward et al., 1991].

A potential VZV vaccine strategy has been devised using hybrid yeast Ty-virus like particles (VLPs) containing VZV gE (amino acids 1-134, 101-161, or 161-233) and assembly protein (AP) inserts as a polyvalent particulate antigen delivery system. VLPs containing viral protein inserts have been shown to be potent immunogens, eliciting both B- and T-lymphocyte ($CD4^+$

Michael Welsh is now at Veterinary Sciences Division, Department of Bacteriology, Stoney Road, Stormont, Belfast BT4 3SD, United Kingdom.

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*Correspondence to: Michael D. Welsh, Veterinary Sciences Division, Department of Bacteriology, Stoney Road, Stormont, Belfast BT4 3SD, United Kingdom. E-mail:-mwelsh@alpha1.dani.gov.uk

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and $CD8^+$) responses [Griffiths et al., 1991; Harris et al., 1992, 1996; Layton et al., 1993; Martin et al., 1993; Gilbert et al., 1997].

The VZV gE is the most abundant virion envelope protein containing important T- and B-cell epitopes [Diaz et al., 1988, 1989; Arvin, 1994; Fowler et al., 1995], and while the AP is believed to be an important immunogen, there have been no reports to date regarding its role in VZV CMI [Harper, 1994; Harper and Grose, 1989]. Antigenicity studies on VZV-VLPs using human VZV convalescent sera have demonstrated strong reactivity with gE(1-134) and gE(101-161) VLPs [Fowler et al., 1995], while some sera reacted with AP-VLPs [Garcia-Valcarcel et al., 1997b]. Immunogenicity studies using gE(1-134), gE(101-161), and AP-VLPs have shown the constructs to prime strong T-cell responses, with the gE-VLPs also inducing VZV neutralizing antibody responses [Garcia-Valcarcel et al., 1997a,b].

Stimulation of PBMCs in vitro with VZV antigen preparations in the lymphocyte proliferation assay (LPA) has been used in many CMI studies [Arvin et al., 1986; Diaz et al., 1988; Giller et al., 1989; Nader et al., 1995; Watson et al., 1990]. This study was performed as a preliminary investigation using the LPA, with the aim of furthering previous VZV-VLP antigenicity work [Fowler et al., 1995; Garcia-Valcarcel et al., 1997b] by determining their ability to restimulate human T-cells primed *in vivo* following natural VZV infection.

MATERIALS AND METHODS

Patient Subjects

Thirty-six consenting adult patients with a recent VZV (varicella or zoster) infection (< 2 months) were referred to our vaccine research study via general practitioners and hospitals within the local health authority. Ethical permission for this work was granted by the East London and The City Health Authority for collection of blood samples from consenting adults over the age of 16 years. Overall 21 patients (varicella, n = 13 and zoster, n = 8) were involved in the study as normal and otherwise healthy patients displaying positive lymphocyte proliferation responses towards VZV-cell lysate antigen (see below). The mean age and time of sample collection post development of rash (PDR) for varicella and zoster patient samples was 34 years (19 days PDR) and 53 years of age (7 days PDR) respectively, with both patient groups having a wide range in age distribution and time of sample collection following infection.

Hybrid Ty-VLP and Cell Lysate Antigen Preparations

VZV gE(1-134)-VLPs, gE(101-161)-VLPs, gE(161-233)-VLPs, AP-VLPs, and control Ty-VLPs without viral inserts were produced as described previously [Fowler et al., 1995; Garcia-Valcarcel et al., 1997b].

To produce VZV infected cell lysate, virus was propagated in MRC-5 cells originally inoculated at a ratio of 1 infected cell to 10 uninfected [Harper et al., 1988] and incubated for 3–4 days until widespread early cytopathic effect was observed. Infected cell layers were rinsed with phosphate buffered saline (PBS) (Oxoid, Basingstoke, U.K.) and scraped into PBS before centrifugation (800g) for 15 minutes at 4°C. Cell pellets were resuspended in 3 volumes of lysis buffer (0.1 M glycine and 0.1 M sodium chloride, pH 9.6) and incubated for 1 hour at room temperature with occasional agitation, followed by ultrasonication . The cell lysate was clarified by centrifugation (300g, 10 minutes) and the supernatant stored at −70°C. Uninfected MRC-5 cells were prepared in the same way as control cell lysate antigen. Protein estimations of antigen preparations were performed using the BioRad protein assay kit-1 (BioRad Laboratories, Ltd., Hertfordshire, U.K.).

Lymphocyte Proliferation Assay (LPA)

Twenty milliliter blood samples were collected into lithium heparin vacutainers (Becton Dickinson Europe, Meylan Cedex, France) and diluted with an equal volume of PBS, before separation of PBMCs over Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden). Serum samples were taken to check for VZV specific IgG and IgM antibodies by immunoblotting, as described previously [Harper et al., 1988].

Following separation, PBMC suspensions were prepared at 2×10^6 cells per ml in complete medium (RPMI-1640 supplemented with 2mM L-glutamine, penicillin 120 μg/ml, streptomycin 200 μg/ml, and 10 mM Hepes buffer) (Gibco, Life Technologies Ltd, Paisley, Scotland) containing 5% foetal calf serum (FCS) (Imperial Laboratories, Ltd., Hampshire, U.K.). FCS batches were screened and selected on the basis of inducing low levels of background cell proliferation in the LPA. To each well of a 96 well microtitre plate 100 μl of cell suspension (2×10^5 cells) was added together with 100 μl of cell lysate or VLP antigens (0.1–10 mg/ml) or concanavalin-A (2.5 μg/ml) (Sigma, Dorset, U.K.) diluted in complete medium. LPA cultures were incubated at 37°C, 5% CO_2 for 6 days before the addition of 0.5 uCi[^{3}H]-thymidine (Amersham International plc, Buckinghamshire, U.K.) to each well for 6 hours. PBMC cultures were harvested onto glass fibre filter paper (Skatron Instruments, Ltd., Berkshire, U.K.) and [^{3}H]-thymidine counts measured by liquid scintillation in Ecoscint (National Diagnostics, Hull, U.K.) on a Canberra Packard scintillation analyser . Results were expressed as stimulation indices (S.I.), which were calculated by dividing test antigen counts per minute (c.p.m) by the relevant control antigen c.p.m. of triplicate cultures.

When sufficient numbers of cells were recovered from blood samples, duplicate LPA sample cultures were prepared and restimulated with antigen at day 6 by replacing 100 μl of culture supernate with the above antigens diluted in complete medium. Antigen restimulated cultures were pulsed with [^{3}H]-thymidine on day 8 and harvested as described above. Statistical analysis was performed using a paired Student's t-test

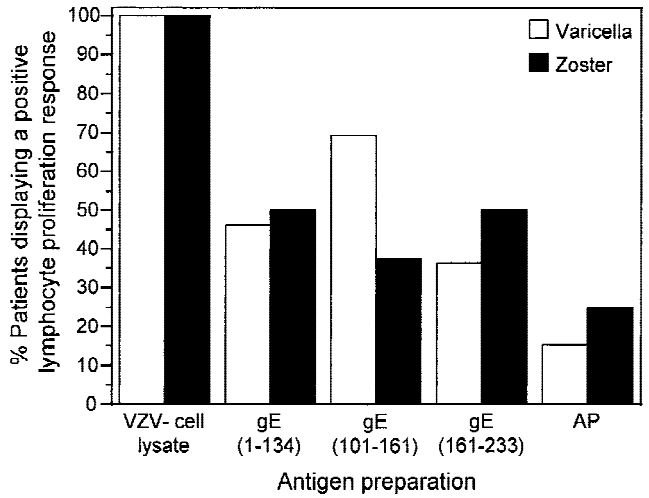

Fig. 1. Percentage of PBMC samples from VZV immune patients, following natural varicella or zoster infection, that proliferated (S.I. ≥ 2) in response to in vitro stimulation with VZV infected MRC-5 cell lysate or recombinant hybrid yeast Ty-VLPs containing VZV protein inserts. S.I. were calculated by VZV test antigen (c.p.m.)/control antigent (c.p.m.).

with a modified degrees of freedom as appropriate for small sample numbers.

RESULTS

Antigenicity of gE-VLPs and AP-VLPs

In order to carry out preliminary laboratory investigations into the antigenicity of hybrid Ty-VLPs containing VZV protein inserts, PBMCs from adult patients with a recent natural varicella or zoster infection were collected and stimulated with antigen in vitro. The LPA was used as a measure of the CMI recognition of VLPs containing gE or AP protein inserts. Cells were stimulated in vitro with either VZV-infected or control cell lysate antigen, or VZV-VLPs along with control VLPs (no VZV protein insert). The standard LPA involved stimulation of the cells with antigen at day 0 with culture harvest on day 6; however when sufficient cells were recovered from blood samples, duplicate cultures were established with restimulation of cells with antigen on day 6 and culture harvest at day 8 (n = 4 for varicella and n = 3 for zoster antigen restimulated cultures).

Proliferation of lymphocytes was measured by [^{3}H]-thymidine incorporation and S.I. for the antigens tested were calculated by dividing test VZV antigen c.p.m for [^{3}H]-thymidine uptake by c.p.m counts for control antigen preparations. The control antigen for the VZV-infected cell lysate was uninfected MRC-5 cell lysate, and the control for the gE- and AP-VLPs was a Ty-VLP without a VZV protein insert. Antigens having S.I. ≥ 2 (with respect to their appropriate control antigen) were considered positive in the LPA.

Figure 1 shows the percentage of patients with a positive lymphocyte proliferation response towards the VZV cell lysate antigen and the individual gE- and AP-VLPs. Overall 85% of varicella and 75% of zoster patient samples had positive proliferation responses towards at least one of the hybrid VZV gE-VLPs, and two patients from each group had S.I. > 2 towards the AP-VLPs. With the exception of gE(101-161)-VLPs, more of the zoster PBMC samples appeared to have a greater capacity to recognise the gE- and AP-VLPs in this study. The Ty-VLPs without inserts marginally elevated the background lymphocyte proliferation to a similar extent as the MRC-5 cell lysate control antigen, compared to the level of proliferation occurring in non-antigen stimulated wells.

The average proliferation responses (S.I.) of varicella and zoster patient samples towards the individual gE- and AP-VLPs together with controls are shown in Table I. Strong S.I. were observed for the VZV-infected cell lysate in the standard 6 day LPA and the antigen restimulated 8 day LPA cultures. Proliferation responses of varicella samples in the 6 day LPA gave low mean S.I. values towards the gE-VLPs and AP-VLPs, with the strongest proliferation responses seen towards the gE(101-161)-VLP, however in the 8 day LPA (antigen restimulation on day 6) the S.I. observed were much greater towards all of the VZV-VLPs including the AP. As seen in Table I the zoster patient samples in 6 day LPAs proliferated more strongly giving higher S.I. values in response to the gE-VLPs, again with the strongest responses seen towards the gE(101-161)-VLPs.

Table II displays the highest S.I. values detected in individual patient PBMC samples towards hybrid VZV-VLPs. It can also be seen in Table II that varicella PBMC samples responded well to restimulation with antigen in 8 day cultures, with S.I. values more than double the values for 6 day cultures. Generally the zoster PBMC proliferation responses diminished by 8 days in culture following antigen restimulation. Statistical analysis revealed significant ($P < 0.05$) proliferation responses towards the VZV-infected cell lysate by both varicella and zoster patient sample groups. With the lower proliferation responses generally induced by the VZV-VLPs compared to the VZV cell lysate and the small patient sample numbers in this study it was not possible to demonstrate statistically significant proliferation responses to the recombinant antigens.

DISCUSSION

Immunization against VZV has been available for many years [Plotkin, 1994; Cimons, 1995], but concerns regarding safety and the long-term protection provided [Clements et al., 1995; Kangro, 1990] have led to the development of alternative vaccine systems, such as recombinant Ty-VLPs containing inserts of VZV proteins.

As the VZV gE protein is highly immunogenic [Diaz et al., 1988, 1989; Arvin, 1994; Fowler et al., 1995], it was expected that PBMCs from VZV immune patients would recognise VZV gE-VLPs. Predominant lymphocyte responses of varicella samples were observed towards gE(1-134) and gE(101-161)-VLPs, both of which contain neutralizing B-cell epitopes [Fowler et al.,

TABLE I. Proliferation Responses of PBMCs From Naturally Infected VZV Immune Patients Following a Primary and a Secondary *In Vitro* Stimulation With VZV-VLPs and Control Antigens

	Mean stimulation indices (S.I.)			
	Varicella patient samples		Zoster patient samples	
Antigen/mitogen	6 day LPA[a] (n = 13)	8 day LPA[a] (n = 4)	6 day LPA (n = 8)	8 day LPA (n = 3)
Control cell lysate	1	1	1	1
VZV cell lysate	7.3[c]	5.4[c]	9.3[c]	4.7[c]
Concanavalin A	10.9[c]	81.7[c]	12.2[c]	36.2[c]
VLP (no insert)	1	1	1	1
gE(1-134)-VLP[b]	1.9	3.6[c]	2.3[c]	1.3
gE(101-161)-VLP[b]	2.2[c]	6.8[c]	4.2[c]	2.7[c]
gE(161-233)-VLP[b]	1.7	4.9[c]	2.5[c]	nt
AP-VLP[b]	1.5	2.9[c]	1.6	1.5

[a]Six day LPA cultures were stimulated with antigen on day 0 and harvested on day 6 and 8 day LPA were stimulated with antigen at days 0 and 6 and then harvested on day 8.
[b]VZV gE protein amino acid sequence inserts or AP insert into recombinant Ty-VLPs.
[c]S.I. of ≥2.0 considered positive. S.I. were calculated by VZV test antigen (c.p.m.)/control antigen (c.p.m.). Control antigens were uninfected MRC-5 cell lysate for VZV infected cell lysate and Ty-VLP without a VZV protein fragment insert for VZV gE- and AP-VLPs.

TABLE II. Maximum Proliferation Responses (S.I.) Detected in Individual PBMC Patient Samples Towards Hybrid VZV-VLPs

	Stimulation indices (S.I.)			
	Varicella patient samples		Zoster patient samples	
Antigen	6 day LPA	8 day LPA	6 day LPA	8 day LPA
gE(1-134)-VLP	3.3[a]	8.8[a]	4.7[a]	1.6
gE(101-161)-VLP	4.5[a]	11.5[a]	10.5[a]	5.3[a]
gE(161-233)-VLP	3.2[a]	6.7[a]	4.2[a]	nt
AP-VLP	2.4[a]	7.2[a]	4.1[a]	1.8

[a]S.I. of ≥2.0 considered positive. S.I. were calculated by gE- or AP-VLP (c.p.m.)/control (no VZV protein insert) Ty-VLP (c.p.m.).

1995]. Zoster samples more readily recognised gE(1-134) and gE(161-233)-VLPs, possibly indicating a change in dominance of VZV epitopes recognised during a zoster reactivation, as has also been observed in B-cell epitope studies [Fowler et al., 1995]. PBMCs proliferated more strongly in the zoster population in 6 day LPAs towards the VZV-VLPs (S.I. values up to 10.5) compared to the varicella population (highest S.I. 4.5), which may correlate with the greater humoral immune response towards VZV antigens observed following zoster than varicella [Harper et al., 1988].

Giller et al. [1989] reported that 50% of PBMC samples from VZV immune patients recognised gpI (gE) (mean S.I. of 9), while in another study only 33% (mean S.I. of 2.2) of PBMC samples from VZV vaccinated patients recognised gpI [Diaz et al., 1988]. In comparison, gE-VLPs contain small fragments (largest insert 134 amino acids) of the 70 kDa gE protein, yet VZV specific CMI responses were detected in 85% of varicella and 75% of zoster patient samples.

The donors in this study may have represented patients with more severe infections as they were submitted to this study through their local doctor or hospital which they attended as a result of the VZV infection. Generally lymphocyte responses to gE-VLPs were lower than expected, possibly indicating that the severity of these infections was a consequence of reduced immunocompetence. Limitations in the ability of adult helper T-cells to respond to VZV [Nader et al., 1995] may explain the low S.I. responses in these varicella patients were the mean patient age was 34 years [Bovill and Bannister, 1998]. The low responses obtained with zoster patients may again be a reflection of age and waning immunocompetence [Berger et al., 1981].

Overall considering the restricted size of the gE protein amino acid inserts (and limited T-cell epitopes) within the VLPs, patient age and immune status, the magnitude of proliferation responses towards gE-VLPs in the LPA compare well with published results using the whole VZV gE protein [Arvin et al., 1986; Diaz et al., 1988; Giller et al., 1989; Watson et al., 1990; Lowry et al., 1992; Sato et al., 1998]. At this stage responses of non-VZV immune patients towards VZV-VLPs are unknown; however control VLPs (no VZV inserts) failed to induce substantial LPA responses in patients from this study, and it has been reported that VZV-antigens do not induce lymphocyte proliferation in non-immune patients and animals [Arvin et al., 1986; Giller et al., 1989; Watson et al., 1990; Lowry et al., 1992; Sato et al., 1998].

CMI responses (particularly $CD8^+$ CTLs) towards the nucleoproteins of many viruses appear to play vital roles in the control of infection. Ty-VLPs carrying nucleoprotein epitopes of Sendai virus and vesicular sto-

matitis virus induce anti-viral CTL responses [Layton et al., 1996]. This is the first report in humans identifying a specific CMI response towards the VZV-AP following both natural varicella and zoster infection. The AP is considered to be a major viral antigen [Harper and Grose, 1989], but our results would not identify it as a dominant CMI antigen, although it remains to determine the role of the AP in relation to protective CTL responses.

Lymphocyte proliferative responses may fall below the level of sensitivity in the normal LPA cultures (6 day) employed in this study; however increased sensitivity could be obtained by restimulating cultures with antigen at day 6. PBMCs from varicella patients responded well to antigen restimulation whereas proliferation responses from zoster patients tended to diminish. This may reflect lower responder cell frequencies in varicella samples towards the antigens under investigation, as indicated by the generally low proliferation responses seen in the 6 day LPAs. Larger sample numbers would have aided in making more accurate conclusions regarding the kinetics of PBMC proliferation responses towards VZV-VLPs tested in the LPA.

Identification of further VZV T- and B-cell epitopes important in protective immunity may allow optimisation of antigens (multiple T- and B-cell epitopes) carried by recombinant VLPs and other vaccine delivery systems [Rajananthanan et al., 1996]. Multiple antigen VLPs carrying up to 15 defined epitopes have been shown capable of priming viral and malarial CTL responses [Layton et al., 1996; Gilbert et al., 1997]. It may also be possible to optimise the epitopes contained within VZV-VLPs to boost protective immunity against herpes zoster [Hayward et al., 1991].

This study has reported on the antigenicity of VZV-VLPs in humans, and together with previous work, demonstrating the ability of VLPs to access both the MHC class-I and -II antigen processing pathways to prime CMI responses, makes the Ty-VLP antigen delivery system a suitable VZV vaccine candidate. Such a vaccine could prove most suitable for boosting VZV immunity prior to immunosuppressive therapy and in other immunocompromised patients. The results obtained in this preliminary study warrant a larger clinical investigation into human cellular responses towards VZV antigens presented in the form of recombinant Ty-VLPs.

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